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Infection With GB Virus C in Leprous Patients in Japan

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The detection of hepatitis C virus (HCV) in blood donors and patients with acute and chronic hepatitis has brought to the fore another virus or viruses which can be transmitted parenterally and induce liver disease. The RNA of a candidate virus designated GB virus C (GBV-C) was determined by the polymerase chain reaction with primers deduced from a helicase-like region in 229 leprous patients in Japan. GBV-C RNA was detected in 12 (5.2%) patients, and HCV RNA in 41 (18%). Three patients were coinfected with GBV-C and HCV. The nine patients infected with GBV-C alone had aminotransferase levels lower than the three patients with the mixed infection or the 38 patients infected with HCV only ($P < 0.001$). Sequence comparison within 100 base pairs in the helicase-like region suggested that two, three and three patients, respectively, would have been infected with three distinct strains of GBV-C. These results indicate that patients with leprosy are at increased risk for infection not only with HCV, but also with GBV-C, and that the infection with GBV-C alone would not induce hepatic injuries as severe as HCV infection.

KEY WORDS: hepatitis viruses, hepatitis C viruses, hepatitis C, chronic hepatitis, leprosy, *Mycobacterium leprae*

INTRODUCTION

The discovery of hepatitis C virus (HCV) by Choo et al. [1989] has enabled the diagnosis of HCV infection by serological and molecular biological means. HCV is responsible for most cases of posttransfusion non-A, non-B hepatitis and acute sporadic as well as chronic hepatitis worldwide. The ability to detect serological and viral markers of HCV infection, however, has revealed a residual risk of posttransfusion hepatitis that is unrelated to HCV or hepatitis B virus (HBV). In addition, there are some patients with acute or chronic hepatitis without serological markers of hepatitis A, B, or C virus infection, who are without evidence for hepatitis D or E virus [Alter and Bradley, 1995].

Viral agents from acute serum from a surgeon (GB) with a retrospective diagnosis of non-A to E hepatitis have been propagated in primates [Deinhardt et al., 1967]. With the advent of molecular representational difference analysis [Lisitsyn et al., 1993], the genomic sequences of two GB viruses were determined and designated GBV-A and GBV-B [Simons et al., 1995b]. Recently, a flavivirus-like genome with sequence similarity to GB viruses was isolated from symptom-free individuals and patients with non-A to E hepatitis, and named GBV-C [Simons et al., 1995a]. The genomes of the GB viruses are positive, single-stranded RNA of ~9,500 nucleotides and can be categorized in the *Flaviviridae* family by their genomic structures; they resemble pestiviruses and HCV, in particular, although with sequences too divergent to be classified as genotypes of HCV [Muerhoff et al., 1995; Simons et al., 1995a,b].

Genomic sequences of GBV-C can be detected by a nested polymerase chain reaction (PCR) with primers deduced from helicase-like region of GBV-C [Simons et al., 1995a]. GBV-C RNA has been detected in four individuals with clinical evidence of hepatitis, one of whom was coinfected with HCV [Simons et al., 1995a]. Furthermore, it has been detected in three of six patients with fulminant hepatitis without evidence of infection with known hepatitis viruses [Yoshiba et al., 1995].

The epidemiology of GBV-C is not clear. The occurrence of non-A to E hepatitis in populations at known risk for HCV infection, such as recipients of multiple transfusion and users of illicit intravenous drugs [Alter

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and Bradley, 1995], may reflect the transmission of causative virus(es), such as GBV-C, in a manner similar to that of HCV. Recently, we reported that patients with leprosy are at high risk for HCV infection [Egawa et al., 1996]. The same cohort of patients was tested for RNA of GBV-C by PCR to see if they were at high risk also for this virus, and to study the sequelae of its infection either alone or in combination with HCV.

MATERIALS AND METHODS

Patients

From July 1993 to June 1994, 200 patients with leprosy were admitted to the National Suruga Leprosarium, as were 29 patients to Koyama Fukusei Byoin in Japan, thus making a cohort of 229 patients. They included 154 males (mean age, 70 ± 9 years, range 45–90 years) and 75 females (70 ± 11 years, 42–97 years). Lepromatous leprosy was diagnosed in 172 (75.1%) patients and tuberculoid leprosy in 30 (13.1%); leprosy in the remaining 27 (11.8%) patients was of a boundary type. The sera were tested for GBV-C RNA by PCR, as well as for markers of HCV and HBV infections and liver function abnormalities. The study was approved by the Ethics Committee of the hospitals, and all the patients gave informed consent.

Determination of GBV-C RNA by PCR

Extraction of RNA and cDNA synthesis. RNAs were extracted from 100 μl of serum using the extraction reagent containing guanidinium isothiocyanate and phenol (ISOGEN-LS from Nippon Gene Co., Ltd., Tokyo, Japan), and dissolved in 5.3 μl of distilled water treated with diethylpyrocarbonate. They were heated at 70°C for 1 min, chilled quickly on ice, and converted to cDNA with reverse transcriptase (Superscript II, GIBCO-BRL, MD) and antisense primer G9 with a sequence of 5′-TCYTTGATGATDGAACTGTC-3′ where, in accordance with the standard IUPAC ambiguity code, Y represented the mixture of T and C; and D did that of A, G and T, which were incorporated into respective sequence positions during the synthesis of oligonucleotide primers.

PCR amplification. Reverse-transcribed cDNA was heated at 95°C for 15 min, and a half portion was subjected to the first round of PCR with TaKaRa Ex Taq DNA polymerase (TaKaRa Biochemicals, Kyoto, Japan) and primers G9 and G8 sequenced 5′-TATGGGCATG-GHATHCCYCT-3′, where H represented the mixture of A, C and T; and Y that of C and T. PCR was performed for 35 cycles (94°C, 30 sec; 55°C, 30 sec; 72°C, 60 sec) followed by an extension cycle at 72°C for 8 min. The second round of PCR was carried out with nested primers G10 (sense, 5′-CATTCVAAGGCGGAGTGYGA-3′ with V representing the mixture of A, C and G) and G11 (antisense, 5′-TCYTTACCCCTRTAATAGGC-3′ with R representing the mixture of A and G). PCR was performed for 30 cycles with each cycle consisting of the same schedule as the first-round PCR, except the primer extension at 72°C which was carried out for 45 sec. The other conditions for PCR were similar to those reported previously for the detection of HCV RNA [Okamoto et al., 1994]. All primer sequences were derived from a helicase-like coding region of GBV-C reported by Simons et al. [1995a]. Expected sizes for products of the first- and second-round of PCR, applied on GBV-C RNA, were 158 and 83 base pairs (bp), respectively, which confirmed the presence of GBV-C genome in the test sera.

For semi-quantification of GBV-C RNA, serial tenfold dilutions of extracted nucleic acids were prepared in distilled water treated with diethylpyrocarbonate and containing 20 μg/ml glycogen (Boehringer Mannheim, Mannheim, Germany), and they were tested by the nested PCR. Relative concentration of GBV-C was expressed by the reciprocal of the highest dilution (10^N) of extracted nucleic aids in which GBV-C RNA was detectable, and it was converted to represent the titer per ml.

Sequence analysis. Products of PCR with primers G8 and G9 were amplified, by a nested PCR, with primers G8 and G11 [Yoshiba et al., 1995]. It was treated with T4 DNA polymerase and T4 polynucleotide kinase, and cloned into M13 phage vectors that had been cleaved with *Hin*cII and dephosphorylated. Sequence of GBV-C cDNA was then determined by the dideoxy-chain termination method with an ALF AutoRead DNA sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). A sequence of 100 bp was determined on three clones each from a serum, and the consensus sequence was adopted for the comparison with GBV-C [Simons et al., 1995a] and against similarly determined sequences of the other sera.

Markers of HCV and HBV Infections

Anti-HCV was sought by enzyme-linked immunosorbent assay (Ortho ELISAII: Ortho Diagnostic Systems, Tokyo, Japan) with the absorbance at 492 nm >0.650 considered reactive. HCV RNA was tested for by PCR with nested primers deduced from the 5′-noncoding region [Okamoto et al., 1990, 1994]. The relative concentration of HCV RNA was expressed by the reciprocal of the highest tenfold dilution (10^N) of extracted nucleic acids in which HCV RNA was detectable, which was converted to represent the titer per ml. Hepatitis B surface antigen (HBsAg) and the corresponding antibody (anti-HBs) were determined by passive hemagglutination with commercial kits (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan) and antibody to hepatitis B core antigen (anti-HBc) by hemagglutination inhibition by the method described elsewhere [Iizuka et al., 1992]. Alpha-fetoprotein was determined by enzyme immunoassay with monoclonal antibodies directed to distinct epitopes on it [Nomura et al., 1983].

Statistical Analyses

Frequency between groups was compared using the χ^2 test and the Fisher's exact test.

RESULTS

Detection of GBV-C RNA in Leprous Patients in Japan

The age-specific prevalence of GBV-C RNA in leprous patients in Japan is shown in Table I in comparison with markers of HCV and HBV infections. Overall, GBV-C RNA was detected in 12 (5%) of 229 patients, at a fre-

TABLE I. Age-Specific Frequencies of GBV-C, HCV and HBV Markers in Leprous Patients in Japan

Age (yrs)	n	GBV-C RNA[a]	Anti-HCV	HCV RNA	HBsAg	Anti-HBs/ anti-HBc[b]
40–59	30	0	4 (13%)	4 (13%)	1 (3%)	12 (40%)
60–69	77	4 (5%)	24 (31%)	14 (18%)	2 (3%)	42 (55%)
70–79	87	6 (7%)	32 (37%)	16 (16%)	2 (2%)	59 (68%)
80–	35	2 (5%)	8 (23%)	7 (20%)	0	15 (43%)
Total	229	12 (5%)	68 (30%)	41 (18%)	5 (2%)	128 (56%)

[a]Determined by nested PCR with primers deduced from a helicase-like region of GBV-C (see Materials and Methods).
[b]Positive for anti-HBs or anti-HBc, or both.

TABLE II. Features of Leprous Patients With GBV-C RNA

Case no.	Age/ sex	Duration[a] (year)	Disease type[b]	Transaminases ALT[c] (IU/L)	Transaminases AST[c] (IU/L)	GBV-C RNA (10^N/ml)	Anti-HCV	HCV RNA (10^N/ml)	HBsAg	Anti-HBs/ anti-HBc[d]	Transfusion
1	64M	45	B	96	83	≥4	2.06	≥4	–	–	–
2	67M	44	L	12	15	1	0.03	–	–	+	–
3	70M	48	L	26	43	2	2.05	≥4	–	+	+
4	76M	49	L	8	15	3	1.96	–	–	+	–
5	88M	35	L	8	16	3	0.05	–	–	–	–
6	95F	42	L	5	17	3	0.03	–	–	+	–
7	72M	45	L	9	12	3	0.05	–	–	+	–
8	61M	49	L	14	10	≥4	1.38	–	–	–	–
9	67M	42	L	9	11	3	0.03	–	–	–	+
10	71M	45	L	25	61	1	2.25	≥4	–	+	+
11	71M	28	L	13	20	1	2.15	–	–	+	–
12	76M	27	L	20	19	1	0.02	–	–	–	–

[a]Duration of hospitalization.
[b]B, boundary type leprosy; L, lepromatous leprosy.
[c]Normal values for alanine aminotransferase (ALT), ≤45 IU/L; aspartic aminotransferase (AST), ≤40 IU/L.
[d]Positive for anti-HBs or anti-HBc, or both.

quency lower than that of HCV RNA detected in 41 (18%) patients. Three patients had GBV-C RNA and HCV RNA, and one patient had HCV RNA and HBsAg, while none had GBV-C RNA and HBsAg.

It is yet to be seen how the frequency of GBV-C infection in leprous patients compares with that in age- and sex-matched controls or the patients with other diseases. In a screening of 60 Japanese blood donors (28 males and 32 females, age 34.9 ± 14.7 years) without elevated levels of alanine aminotransferase (≤45 IU/L), GBV-C RNA was not detected in any sera. Hence, the prevalence of GBV-C infection among the general population in Japan would not be high and much lower than that in leprous patients, 5% of whom were infected.

GBV-C RNA was detected in six (8.8%) of 68 patients with anti-HCV, and three of them were positive for HCV RNA in serum. By contrast, it was detected in six (3.7%) of 161 patients without anti-HCV. Thus, GBV-C RNA was twice more common in patients with anti-HCV than in those without; the difference fell short of being significant, however.

The features of the 12 patients with GBV-C RNA in serum are shown in Table II. Males predominated and accounted for 92% with a prevalence somewhat higher than that in the patients with HCV RNA (28/41 or 68%) or the total patients studied (154/229 or 67%). The majority (11/12 or 92%) had lepromatous leprosy, in comparison with 172 (75%) of 229 studied patients who had this type of leprosy. None of the 30 patients with tuberculoid leprosy had GBV-C RNA, while one (4%) of the 27 patients with boundary type leprosy did.

Elevated transaminase levels were detected in three patients with GBV-C, all of whom were coinfected with HCV. None of the remaining nine patients who had GBV-C RNA unaccompanied by HCV RNA had elevated transaminase levels.

Comparison of Patients Infected With GBV-C, HCV, or Both, or Neither

The 229 leprous patients were classified into four groups by GBV-C and HCV infections. Table III compares the features of: (1) nine patients with GBV-C RNA alone; (2) three with GBV-C RNA as well as HCV RNA; (3) 38 with HCV RNA alone; and (4) the remaining 179 without GBV-C RNA or HCV RNA. There were no appreciable differences in their age, years of hospitalization, or serological markers of HBV infection. The nine patients who were infected with GBV-C alone had levels of alanine and aspartate aminotransferases comparable with those in the patients without GBV-C RNA or HCV RNA, which were significantly lower than those in the patients infected with HCV either alone or together with

TABLE III. Features of Leprous Patients Who Were Infected With GBV-C or HCV, or Both, or Were Not Infected With Either

Features	Detection of GBV-C RNA and HCV RNA			
	GBV-C (+) HCV (−)	GBV-C (+) HCV (+)	GBV-C (−) HCV (+)	GBV-C (−) HCV (−)
Number	9	3	38	179
Age	75 ± 10	68 ± 3	71 ± 9	70 ± 10
Male (%)	8 (89%)	3 (100%)	25 (66%)	118 (66%)
Type[a] L	9 (100%)	2 (67%)	29 (76%)	132 (74%)
T	0	0	7 (18%)	23 (13%)
B	0	1 (33%)	2 (5%)	24 (13%)
Duration (year)	40 ± 8	46 ± 2	38 ± 14	39 ± 10
Transfusion	1 (11%)	2 (67%)	3 (8%)	6 (3%)
Anti-HCV	3 (33%)	3 (100%)	38 (100%)	24 (13%)
HBsAg	0	0	1 (2.6%)	4 (2.2%)
Anti-HBs/c[b]	5 (56%)	2 (67%)	20 (53%)	101 (56%)
ALT				
Raised	0	1 (33%)	11 (29%)	4 (2%)
Mean (IU/L)	11 ± 4	49 ± 33	48 ± 58	15 ± 11
AST				
Raised	0	3 (100%)	17 (45%)	4 (2%)
Mean (IU/L)	15 ± 3	62 ± 16	54 ± 59	19 ± 16
AFP[c]	0	0	7 (18%)	NT[d]

[a]L, lepromatous; T, tuberculoid; B, boundary type.
[b]Positive for anti-HBs or anti-HBc, or both.
[c]Alpha-fetoprotein elevated (>6.9 ng/ml).
[d]Not tested.

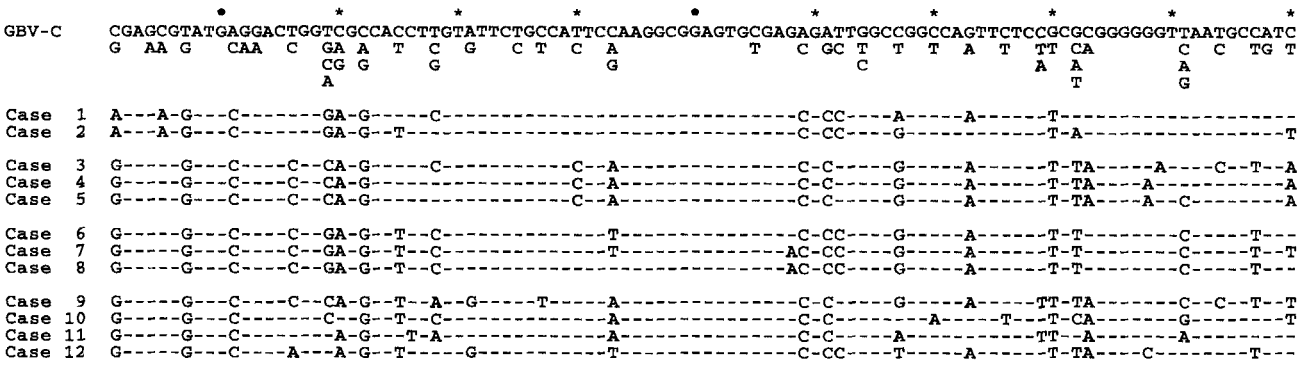

Fig. 1. Nucleotide sequence of a part of GBV-C from the patients with leprosy. The original sequence of GBV-C and variation seen in 7 other isolates reported are indicated above [Simons et al. 1995a]. The consensus sequence of three clones each from the 12 patients are shown below.

GBV-C ($P < 0.001$). All the seven patients with elevated levels of alpha-fetoprotein were in the group of patients with HCV RNA unaccompanied by GBV-C RNA in serum.

Nucleotide Sequences of GBV-C From Leprous Patients

Among 3 clones each from sera of the 12 patients, nucleotide sequences of 100 bp, in the helicase-like region of GBV-C, differed only by ≤ 2 bp, none of which induced amino acid changes: 0 bp for cases 5, 7, 8, and 11; 1 bp for cases 3, 6, 9, and 10; and 2 bp for cases 1, 2, 4, and 12. Figure 1 compares the consensus sequences of GBV-C from patients with leprosy among themselves and against the sequences reported by Simons et al. [1995a]. Sequences from the patients were similar to those of Simons et al. [1995a] sharing 76–87% of the 100 nucleotides in comparison. Some resemblance was noted among sequences from the 12 patients. The patterns of point mutations suggested that two (cases 1 and 2), three (cases 3–5), and three (cases 6–8) patients, respectively, would have been infected with 3 distinct strains of GBV-C.

DISCUSSION

Patients with leprosy have been known to be at high risk for HBV infection [Blumberg et al., 1967]. In a previous study [Egawa et al., 1996], they were also found at increased risk for HCV infection in confirmation of previous reports [Denis et al., 1994; Frommel et al., 1993]. Leprous patients have been confined for a prolonged period traditionally and many have open skin

lesions, which would be among reasons for horizontal transmission of these hepatitis viruses. Our patients have been institutionalized for up to 50 years, and most had received intravenous drugs regularly for treating pain with nondisposable syringes and needles in the long past, which might have contributed to the spread of HCV.

Whatever the mechanism of HCV transmission among leprous patients, it is also similar for GBV-C. Using PCR with nested primers deduced from a helicase-like region [Simons et al., 1995a], GBV-C RNA was detected in 12 (5%) of 229 patients, three of whom were coinfected with HCV. The prevalence of GBV-C RNA was less than that of HCV RNA which was detected in 41 (18%) patients. At present, it is not certain if the prevalence of GBV-C infection in leprous patients was higher than that in the general population in Japan, although it was detected in none of 60 Japanese blood donors without elevated levels of alanine aminotransferases. Nonetheless, the results obtained clearly indicate that GBV-C would be transmitted in a similar manner as HCV, but that it would be less infectious than HCV. Surveys for GBV-C RNA in other populations at high risk for HCV, such as patients with hemophilia and those with chronic renal failure treated by maintenance hemodialysis, as well as individuals with intravenous drug abuse, would be required to verify this view.

Although a 100-bp sequence is too small a region of comparison, sequences of GBV-C isolates from the 12 patients with leprosy suggested that some of them might have been infected with the same strain of the virus. This would reflect a horizontal infection of GBV-C within a closed society, and a common-source infection in institutions. None of the isolates, however, had the same sequence within the 100 bp in comparison. This would mirror rapid evolution of GBV-C, a characteristic inherent to RNA viruses such as HCV.

Despite the association with non-A to E fulminant hepatitis [Yoshiba et al., 1995], it is not known what would be the sequel of infection with GBV-C. The leprous patients infected with GBV-C alone did not have elevated levels of serum aminotransferases. Insofar as a long history of GBV-C infection is reasonably presumed for most of them, this putative non-A to E hepatitis virus would not induce liver damage as severe as HCV. This view is supported by a mild clinical course of non-A to E hepatitis observed in epidemiological studies [Alter and Bradley, 1995]. The ability of GBV-C to induce hepatic damage awaits further studies.

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